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STUDIES ON A FUNGOUS "DIE-BACK" DISEASE OF THE MOUNTAIN ASH

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STUDIES ON A FUNGOUS "DIE-BACK" DISEASE OF THE MOUNTAIN ASH

Stanley Burnham Clay
Department of Field Crops

A THESIS
submitted to the University of Alberta
in partial fulfilment of the requirements for
the degree of
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This thesis represents one-half of the total work

Edmonton, Alberta
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STUDIES ON A FUNGOUS "DIE-BACK" DISEASE OF THE MOUNTAIN ASH

Stanley Burnham Clay

INTRODUCTION

The mountain ash is one of the more popular and one of the most beautiful of the ornamental trees grown in western Canada. Species grown here are generally considered as being long lived and hardy in this climate and, until recently, they have apparently been subject to relatively little damage from disease. In 1936, however, it was observed that a large number of trees in Edmonton were affected with a "Die-Back" disease. This caused considerable alarm to property owners on whose premises blighted trees occurred. Since the disease appeared to be of sufficient importance to warrant an investigation, the studies here reported were undertaken.

A preliminary survey indicated that the disease in question closely resembled a "Die-Back" of poplars and willows reported in the United States by Long (11) and Povah (13) and found to be caused by species of fungi belonging to the genera Valsa and Cytospora. The Edmonton survey indicated that similar fungi are consistently associated with the blight of mountain ash.

Since the majority of the mountain ash trees growing in western Canada are European mountain ash (Sorbus aucuparia), an introduced species, it seemed probable that these trees might be especially susceptible to attack by Valsa and Cytospora species whose natural habitats are the dead branches of poplars, willows and other native trees. This suggestion is, in fact, in keeping with observations on other tree diseases. Boyce (2), for instance, in a discussion of the value and limitations of introduced tree species in the United States, says, "An introduced tree is likely to find among our fungi at least one parasite to which it will lack resistance." To substantiate this statement Boyce gives several examples, among which is the case of the Himalayan pine introduced into Michigan which "after 10 years was so severely diseased by a canker, with which Valsa superficiales Nitschke was associated, that ultimate destruction of the plantation was certain."

The fact that Cytospora chrysosperma has been reported as causing a disease of poplars in Alberta (15), and is found very frequently in Edmonton on diseased poplars, suggested that this host, or other tree species, might be the source of inoculum for the mountain ash. The probable relations of the disease on the mountain ash to similar diseases of poplars and other trees were considered to be of sufficient importance to merit special attention in connection with a study of the disease of the mountain ash.

The investigations undertaken included (1) a study of the disease as it occurs on the mountain ash; (2) a study of the relations of this disease to similar diseases on other trees, especially the poplar; and (3) a study of the physiology and pathogenicity of fungi found associated with these diseases.

It was hoped that following a careful investigation of the disease and factors affecting it, satisfactory preventive and control measures might be suggested.

The experiments and observations reported in this paper were conducted during the interval from the spring of 1937 to the spring of 1939. Practically all the work has been done and the observations made in the city of Edmonton.

HISTORICAL

Although species of *Valsa* and *Cytospora* were named as early as 1823 (6), when they were reported as occurring on dead twigs, it was not until comparatively recently that these fungi were proved to be definite pathogens. Saccardo (14) reports species of *Valsa* and *Cytospora* growing on many trees, including *Sorbus* spp., in his "*Sylloge Fungorum*", 1882. During the first 20 years of the present century investigations into the importance of *Cytosporeae* and *Valsaceae* as causal agents of disease were begun, and it was during this period that the pathogenicity of these fungi was definitely established. Some of the more important papers dealing with the conditions under which these fungi become pathogenic and with host parasite relations will be reviewed.

Aderhold (1), one of the first workers in this field, carried out extensive investigations on the morphology, taxonomy and pathogenicity of *Valsa leucostroma*, including experiments with pure cultures. By extensive cultural and inoculation studies he demonstrated the parasitism of this fungus on drupes and also its ability to cause disease in the apple. Another of the early papers dealing with *Cytospora* diseases of *Rosaceae* is that of Stevens (17) which reports the results of an investigation in 1919 of an apple disease. Stevens worked with an apple canker caused by a species of *Cytospora*, and he

considered the fungus to be a dangerous pathogen. Because he could not produce the perfect stage in culture, he did not attempt to name the species. This paper includes an extensive bibliography. Leonian (9) in 1921, working with a species of *Valsa* from apples resembling *V. leucostroma*, found that inoculation of healthy apple trees did not produce disease. He observed, however, that apple trees weakened by the wooly aphis working in the roots, or by the giant apple borer girdling the underground part of the trunk, became infected and were soon killed by the fungus. As a check on species identification, Leonian also inoculated with *Cytospora chrysosperma*, and found that pure cultures of this fungus were unable to infect any of the apple trees. Fischer and Reeves (5) in 1929 proved by inoculation experiments that *Cytospora* spp. were only weak wound parasites on the apple and that diseased trees could recover if soil and environmental conditions became favorable. The trees were attacked only when growing under unfavorable conditions, and it was only under these conditions that the disease became destructive. They found that trees which had been attacked in 1927 recovered when sufficient water, fertilizers and good soil were provided in 1928.

On other than rosaceous hosts the most important work done with species of *Cytospora* and *Valsa* has been on poplars and willows. Long (11) in 1918 described a serious die-back of poplars and willows as being caused by *Cytospora chrysosperma*. In a discussion of factors

favoring disease production by this fungus, Long outlined the following four general conditions under which disease may occur:

(1) Trees growing at the outer limits of their range and, therefore, in a more or less unfavorable environment are liable to be more susceptible.

(2) Trees planted in streets, lawns and cemeteries where they have become weakened from neglect and lack of water are apt to be attacked by *Cytospora* spp.

(3) Disease caused by *Cytospora* is liable to be severe in trees weakened by extensive pruning as in pollarding.

(4) Cuttings in propagation beds are liable to become diseased when the usual methods of propagation are used.

Trees planted in shelter belts and windbreaks in the arid and semi-arid regions of the southwestern United States were studied by Long and were included in the first class listed above.

Povah (13), in 1921, found that *Cytospora chrysosperma* was important in killing trees that had been injured slightly but not fatally by light ground fires at Syracuse, New York. He also reported *Valsa sordida* as being present on the same trees, and suggested the possibility that this might be the perfect stage of *C. chrysosperma*, an hypothesis which was later proved to be correct by Schreiner (16) in 1931.

In Canada the first important reference to a disease caused by C. chrysosperma was that of Moss (12) in 1921. Moss reported C. chrysosperma as occurring on Populus alba, P. deltoides and Lombardy poplars in Ontario. Some of the poplars were killed from the top, branch by branch, and some had cankers near the base. Moss concluded that the fungus was important as a factor causing the death of poplars in parks and woodland in Ontario.

A careful search through all available literature revealed no report of previous experiments with Cytospora spp. as causal agents of disease of Sorbus spp., although the presence of Valsa and Cytospora spp. on these trees has been reported several times (14).

The only report of Cytospora spp. causing a tree disease in Alberta is that of Sanford (15) who in 1931 reported a disease of poplars caused by C. chrysosperma as being more or less secondary to weakening of the trees by adverse environmental conditions.

In general, these investigators came to the conclusion that species of Cytospora are at most only weak wound parasites, and that before they can cause serious disease the host must be predisposed by injury, adverse environmental conditions or some other factors.

SYMPTOMS AND SIGNS OF THE DISEASE

Symptoms

In the most frequently found form of the disease the branches die from the tips downward, and these dead limbs stand out in contrast to the normal leafy branches of the trees (Figure 1). An extreme case is illustrated in Figure 2. An affected branch commonly dies down to its juncture with a larger branch or with the main trunk during the first season, and the disease may then be arrested. If the disease is not arrested, it spreads and develops in the larger branch or trunk, forming a more or less circular canker around the base of the infected branch. In the cases where the disease has been arrested it is thought that the trees have been attacked while subjected to adverse environmental conditions, and that with the return of a more favorable environment, the trees regained their natural vigor and have been able to prevent further progress of the disease.

In a less frequently found form of the disease, lesions or cankers of the trunks may occur subsequent to injury or following sunscald. These cankers gradually enlarge until eventually they may girdle and kill the tree. Figure 3 illustrates a form of sunscald which often precedes this disease, and which is common in Edmonton.

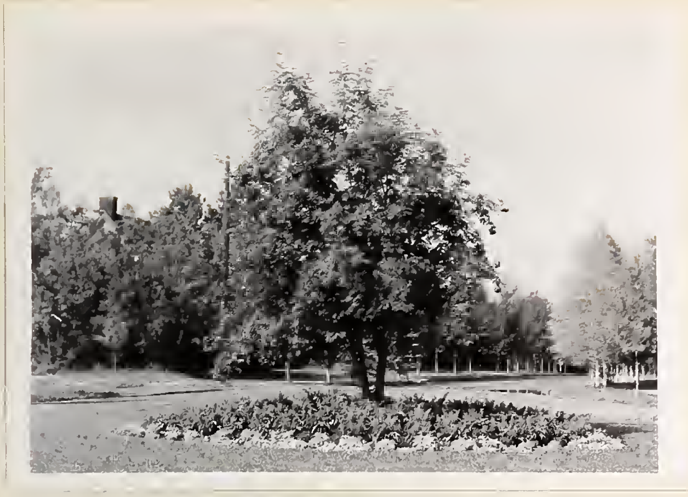

Figure 1

Mountain Ash, Sorbus aucuparia.
Typical healthy tree.

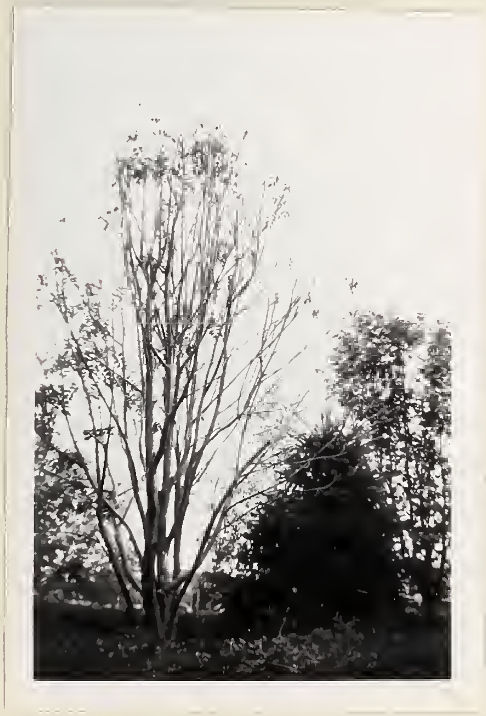

Figure 2

Diseased Mountain Ash showing the
"Die-Back" type of infection

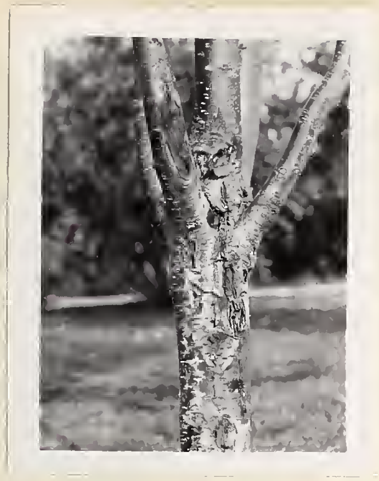

Figure 3

Sunscald canker of the Mountain Ash
which has become infected with Cytospora

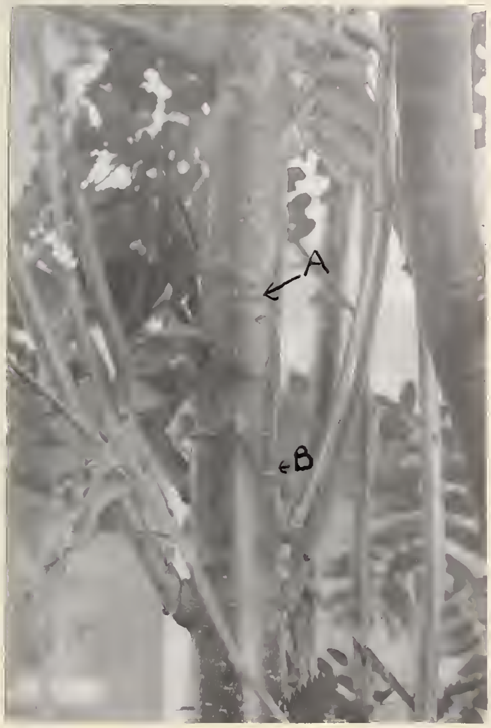

Figure 4

Progressing "Die-Back" lesion showing perithecia of *V. ambiens* above, A, which is the portion of the canker produced prior to and during 1937, and the new portion of the canker from A to B produced in 1938

One of the most obvious features of the lesions produced in this disease is the sharply defined dividing line between the diseased and healthy tissue (Figure 4). The diseased part of a branch not only differs in color and bears numerous fungal fruiting bodies, but also is slightly sunken as compared with the adjacent healthy part.

Signs of the Disease

Pycnidia or perithecia of fungi belonging to the genera *Cytospora* and *Valsa* appear on the diseased tissue, in both the die-back and the trunk canker form of the disease, soon after affected parts begin to die. These fruiting bodies form numerous tiny elevations in the bark of the dead branches to within a centimeter or two of the margin of the cankers.

The fruiting bodies near the advancing margins of the cankers are pycnidia, at least in all cases that have been examined, while those in the older parts of the cankers may or may not be perithecia. Both pycnidia and perithecia are slightly raised conically above the surrounding surface about 1 mm. and are 1-2 mm. in diameter (Figures 5 and 6). Perithecia can often be identified by their larger size and by the presence of a dark ring about 2 mm. in diameter encircling each (Figure 6). Such rings, however, are also present around certain pycnidia (Figure 5) so they cannot always be taken as an indication of perithecia.

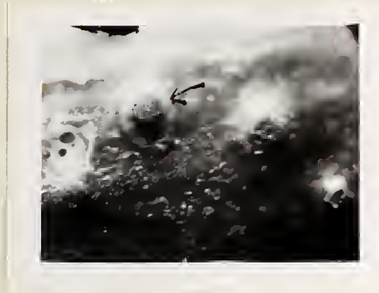

Figure 5

Pycnidia of C. ambiens with characteristic dark ring marked by the tip of the arrow (Magnification 6 times)

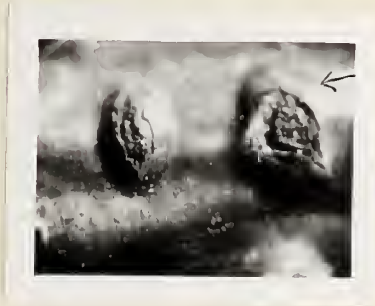

Figure 6

Perithecia of V. ambiens. Dark ring marked by the tip of the arrow does not show plainly in this photograph (Magnification 6 times)

Two types of pycnidia were commonly found associated with diseased twigs: (1) those just mentioned which are surrounded by dark rings and are fairly widely scattered, that is, few per unit area; and (2) those occurring in dense aggregations and tending to darken the host epiderm right up to their summits. Because this second type of pycnidium gives the diseased branch a blackened appearance it will be referred to in the following discussion as the "black type of pycnidium". The latter is found much more frequently in association with the disease of the Mountain Ash than is the dark ringed type.

The color of the cankers or diseased tissue varies from a tan to a dark brown, depending on the species of fungus present and on the part of the tree affected. Diseased tissue bearing the fungus with black pycnidia is darker than tissue infected with the ringed species. Moreover, cankers on trunks and large branches are darker than cankers on small twigs or small branches.

THE PROGRESS OF THE DISEASE ON THE TREE

Although the disease often advances a certain distance each year, this progression does not take place at a uniform rate throughout the season. Cankers examined during the summer appear to be dormant and to be held in check by a ~~callus~~ cork layer which sharply separates the diseased from the healthy tissue. The sharp line between diseased and healthy tissue at B in Figure 4 is formed in part by this ~~callus~~ cork layer. The disease seems to be able to progress beyond this ~~callus~~ cork barrier in the spring and to advance a certain distance, but it becomes walled off again during the summer and ceases to enlarge. One season's advance is illustrated by the area from A to B in Figure 4. The line of demarcation for the previous season (1937) can be observed at A in the photograph, and the 1938 season's advance is represented by the distance from A to B. Observe the new branches just below the canker which, with the remainder of the branch, may eventually be killed. Perithecia of a Valsa species can be quite easily observed in Figure 4 on the older canker above A, and pycnidia are forming on the new portion of the canker from A to B.

FUNGI ASSOCIATED WITH THE DISEASE

By removing small pieces of diseased tissue from the advancing edges of cankers, surface sterilizing these pieces and plating them on potato dextrose agar, fungi in pure culture were invariably obtained. These fungi grew out rapidly on to the agar and soon produced pycnidia and spores characteristic of the genus Cytospora. These isolations in pure culture, together with results of examinations of fungal fruiting bodies found on the diseased trees, established the fact that Valsa and Cytospora species are associated with both the die-back and the trunk canker forms of the disease.

A detailed study of the pycnidia and perithecia found on the trees and of cultural characters of the isolates grown on potato dextrose agar was undertaken in an attempt to identify the fungi associated with the disease.

The isolations from the edges of the cankers and those from pycnospores fell into two groups as to cultural characters on potato dextrose agar: (1) a fast growing group producing mycelium which varied in color with age from greenish to dark green; and (2) a slow growing group characterized by a whitish to pinkish mycelium. A photograph of these fungi growing in culture is presented in Figure 15 (under cultural studies). Cultures were numbered progressively in order of their isolation with no attempt to separate the groups.

While attempting to identify the *Valsa* found on the older part of the lesions, it was observed that isolates of this *Valsa* grown on potato dextrose agar resembled isolates of the slow growing group with white mycelium obtained from pycnidia. By culturing these isolates on mountain ash twigs in sterile flasks, it was found that both the *Valsa* isolates and those of the slow growing group produced pycnidia encircled by dark rings (Figure 5). By collecting fresh specimens from diseased trees and making isolations from perithecia with black rings and from pycnidia with black rings, it was found that the cultures obtained were identical and fell into Group 2, the one with white mycelium. Before these relations were known, specimens of *Valsa* with dark-ringed perithecia and of *Cytospora* with dark-ringed pycnidia were identified by Dr. Dearness* as *Valsa ambiens* and *Cytospora ambiens* respectively. It seems, therefore, that enough evidence has been obtained to establish definitely the identity of this slow growing type, which produces ringed pycnidia, as *Valsa ambiens*, the conidial stage of which is *Cytospora ambiens*. During the remainder of this paper the slow growing group with white mycelium will be considered as being composed of isolates of *Valsa ambiens* and will be so named.

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* The writer wishes to acknowledge his indebtedness to Dr. John Dearness, Mycologist, London, Ontario, for identification of the fungi and for useful suggestions which were of considerable aid in this study.

It seemed to follow from the proof of the relation between the Valsa ambiens group and the ringed pycnidia, that the isolates contained in Group I, that is, fast growing strains with greenish mycelium, are associated with the black pycnidia. This was proved: (1) by isolating typical Group I strains from black pycnidia; and (2) by obtaining black pycnidia on sterilized twigs after Group I strains had been cultured on them for about two weeks.

The specific identification of this group was much more difficult in that no perfect stage could be found associated with it. During investigations reported under "Sources of Fungi Found Associated with the Disease" it was discovered that cultures on potato dextrose agar of a Valsa sp., which inhabits dead branches of the saskatoon (Amelanchier alnifolia), and cultures on the same medium of Group I from the mountain ash were identical. Black pycnidia, typical of Group I, were produced when sterile twigs of mountain ash were inoculated with the Valsa sp. from the saskatoon. These findings were considered as strong evidence of genetic relationships between the Valsa sp. found on the saskatoon and the Cytospora sp., which produces black pycnidia and is designated as Group I, found on the mountain ash. The Valsa sp. from the saskatoon was identified by Dr. Dearness as "at or near Valsa cincta". It is therefore believed that the Cytospora species which produces the black pycnidia of Group I on the mountain ash

is *Cytospora cincta*, the imperfect stage of *Valsa cincta* which inhabits the saskatoon. A more detailed account of this relationship is given under "Sources of Fungi Found Associated with the Disease".

HISTOLOGICAL STUDIES

Histological studies were made of both the perithecial and pycnidial stromata found on the mountain ash to help in the identification of the fungi and to elucidate, if possible, any relations that might exist between the Valsa and Cytosporeae present.

Several techniques for embedding and sectioning were tried, and it was found that for woody tissue such as bark and fungal stroma, Zirkle's (19) N-butyl alcohol method was most satisfactory. The Dioxane method (Stain Technology 12(4), 1937) and the ethyl alcohol procedure of Chamberlain (3) left the wood unduly hardened. Staining was done with safranin and light green in clove oil, following Chamberlain's method (3) for paraffin sections. This procedure leaves fungal spores and ligneous tissues of the wood stained red and fungal hyphae and parenchymatous tissue of the wood stained green. These colors were satisfactory for photographic purposes when the proper light filters were used.

The Pycnidial Stromata

A photograph of a vertical section of a pycnidium is shown in Figure 7. This pycnidium is representative of both groups found on the mountain ash. Pycnidia of the V. ambiens group, characterized by a dark ring at (B), can

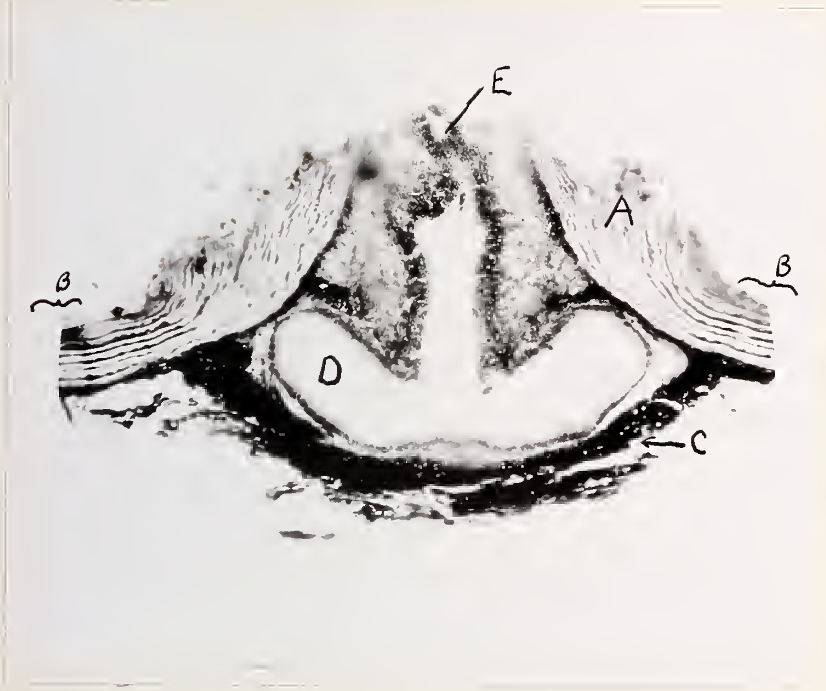

Figure 7

Pycnidium of Cytospora ambiens.
Vertical section.

A. Ruptured host bark.
B. Position of dark circle.
C. Basal stroma.
D. Locular chamber.
E. Ostiole.

(Magnification 80 times)

also be differentiated from those of the other group by the fact that they have much less dense basal stromata (C). Moreover, branching of the conidiophores is more profuse and the conidiophores are a little longer in _C_. _ambiens_ than in the other group. The average length of the conidiophores in the former was found to be 19-28 microns as compared with 15-25 microns in the latter.

The pycnidia are approximately the same size in both groups, measuring 0.8 to 1.0 mm. in diameter through the radii of the locular chambers and 0.8 to from 1 to 2 mm. in height from the basal stroma to mouth of ostiole. Variations in size and shape occur, depending on the nature and thickness of the bark in which they are borne.

The Perithecial Stroma

Valsa _ambiens_ was frequently found on infected twigs and limbs on the older parts of the lesions. Histological studies of the perithecial stromata were made to aid in the identification of the fungus and to determine the relation of perithecia to the pycnidia in the stromata.

Vertical and horizontal sections of a perithecial stroma are shown in Figures 8 to 12. Figures 8, 9 and 10 show quite clearly that the perithecia (C) are arranged in a circle below and to the outside of a pycnidium (A). It is also apparent that the perithecia lie directly below the dark circle (B) mentioned previously. It is quite

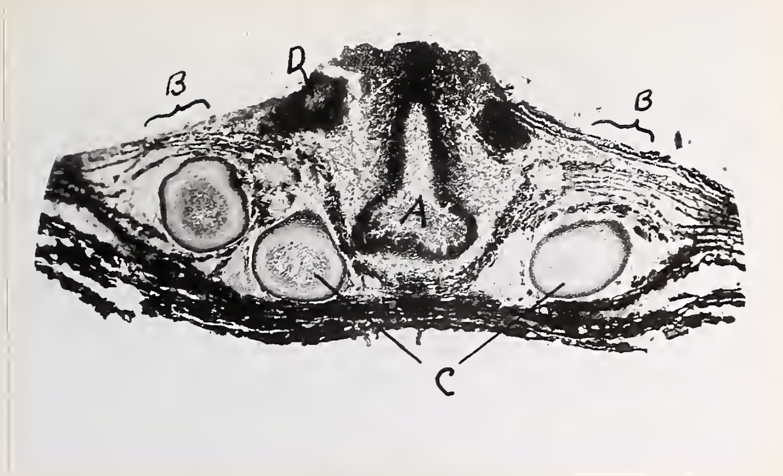

Figure 8

Perithecial stroma of _Valsa_ _ambiens_.
Vertical section.

A. Pycnidium
B. Position of dark circle
C. Perithecia
D. Ostiole of a perithecium

(Magnification 52 times)

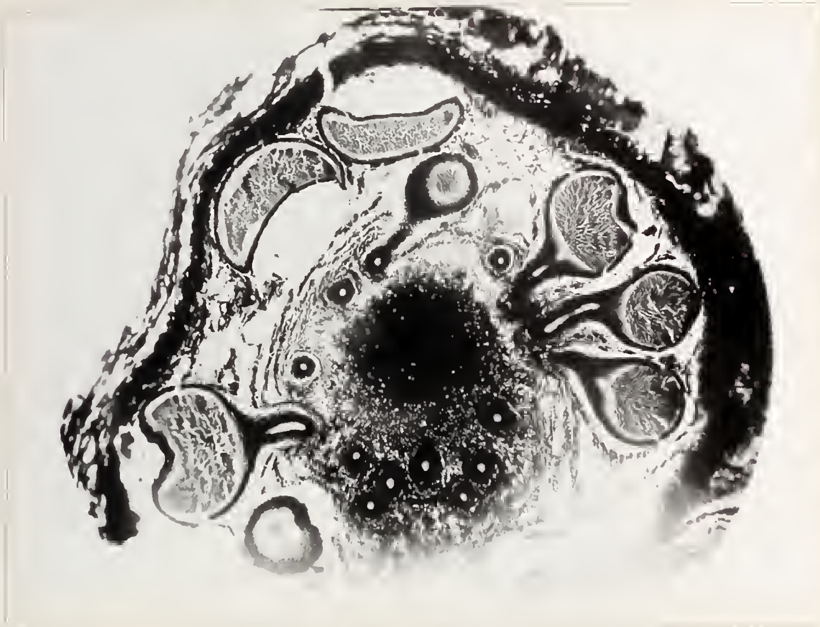

Figure 9

Perithecial stroma of _Valsa_ _ambiens_.
Horizontal section.

Note the arrangement of the perithecia and the ostioles of perithecia in a lower stratum.
(Magnification 52 times)

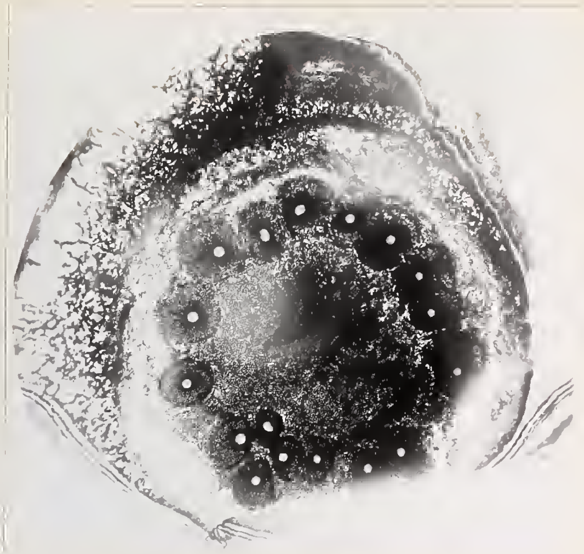

Figure 10

Perithecial stroma of Valsa ambiens.

Horizontal section just below the summit of the pustule to show the arrangement of the ostioles of the perithecia.

(Magnification 52 times)

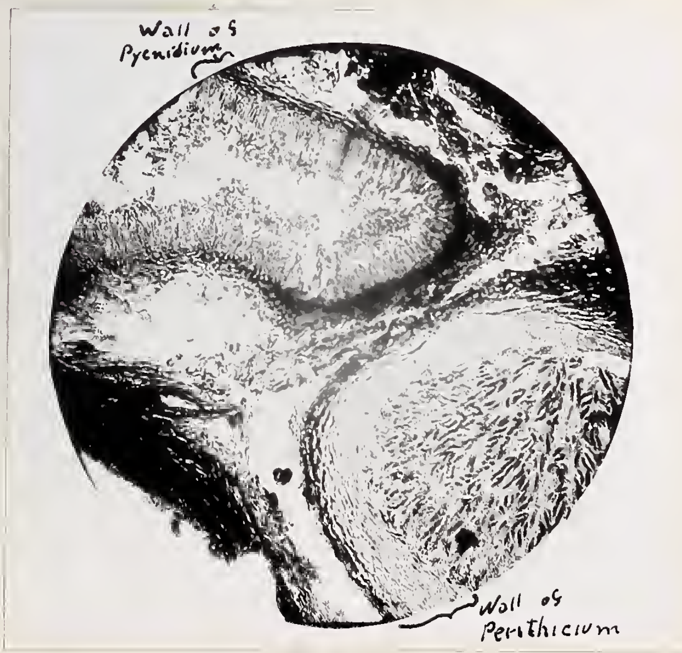

Figure 11

Perithecial stroma of Valsa ambiens.

Enlarged section of stroma showing a comparison of the perithecial and pycnidial walls.

(Magnification 144 times)

conceivable that there is a relation between this dark circle and the perithecial primordia even in Cytospora ambiens when the actual perithecia are not found. The ostioles of the perithecia converge towards the centre as they rise to the summit to form a close ring around the ostiole of the pycnidium, Figures 8 (D), 9 and 10, and it is from this ring that the specific name ambiens is derived.

The perithecium is 0.35 mm. in diameter on the average, with a neck 0.7 mm. to 1.3 mm. in length, slender and thickened at the ostiolar end. It has a thin deeply staining wall, and is filled with asci (Figures 11 and 12).

A comparison between the pycnidial wall and the perithecial wall is shown in Figure 11 where a section of a pycnidium adjacent to a section of a perithecium is shown in the same photograph. The conidiophore layer can be quite easily seen lining the pycnidium; the pycnospores can be observed in the cavity. The perithecium, on the other hand, has a thin leathery outer wall and a parenchyma-like inner wall, and is densely crowded with asci (see also Figure 12) amongst which no paraphyses have been observed.

Asci can be seen in much better detail in Figure 13 where they are mounted unstained in water, and Figure 14 where they are unstained in India ink. Asci are most commonly 8-spored and measure 50-60 microns long, averaging 53 microns, and 7-10 microns wide, averaging 8-9 microns. Ascospores are 12 to 18 microns long and 2-3 microns in diameter.

The reason for the occurrence of the perithecia in

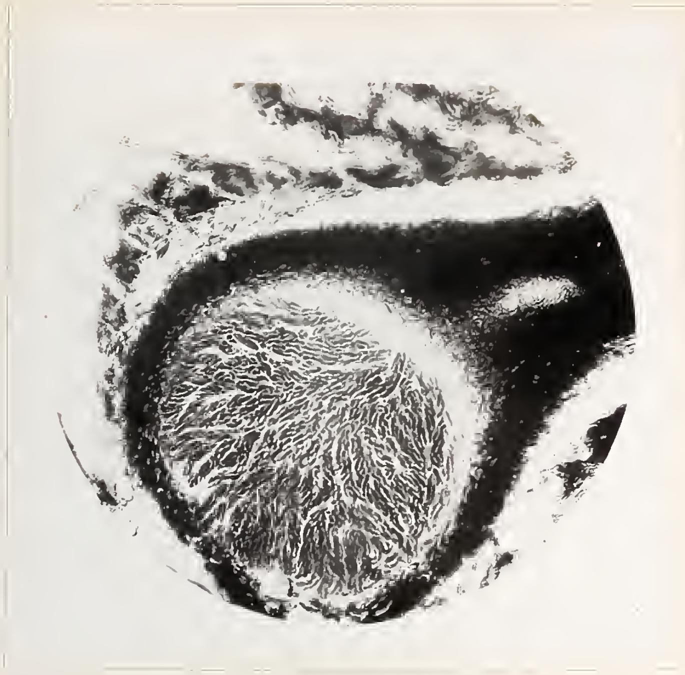

Figure 12

Perithecium of Valsa ambiens.

Note thick leathery wall and contained asci.

(Magnification 144 times)

Figure 13

Asci. Water mount unstained.

(Magnification 340 times)

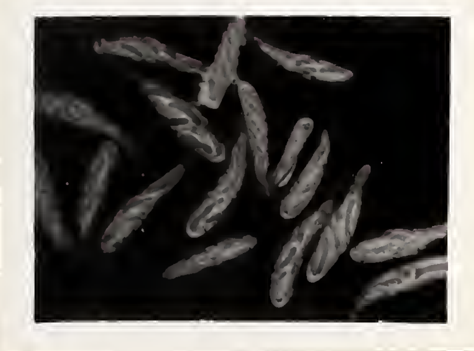

Figure 14

Asci. India ink mount unstained.

(Magnification 340 times)

such a regular manner about the base of the pycnidium and factors influencing their initiation and development would make an interesting mycological and genetical investigation. Such a study is beyond the scope of this paper.

INOCULATION STUDIES

During the spring and summer of 1938 inoculations were made on the Sorbus, Salix and Populus with Cytospora spp. isolated from all three of these hosts. The objects of this investigation were: (1) to determine the pathogenicity of each fungus to the host from which it was isolated; and (2) to determine the pathogenicity of each fungus to trees belonging to other of the above-mentioned genera than that from which it was originally obtained.

The results presented in this section are for one year only.

Materials and Methods

Fungi.

Only isolates from severely infected trees were used for inoculation purposes because it was thought that the fungi invading these trees might be especially pathogenic. The cultures used consisted of the 15 isolates from mountain ash, 7 isolates from poplar and 4 isolates from willow. Isolates 7, 10, 11 and 12 from the mountain ash represented the Valsa ambiens group, and the other 11 of the 15 were members of the fast growing group. One isolate from Prunus virginiana (choke cherry) was also included because it seemed to be especially virulent on a group of severely diseased choke cherries from which it was

obtained.

Trees.

Mountain ash trees used in the inoculation studies consisted of two groups: one from ~~the~~ Taylor's Nursery* of Edmonton which was transplanted from the nursery to boxes and grown outside in the summer and in the greenhouse in the winter, and a second group of six trees growing in the orchard of Mr. G. Harcourt*, adjacent to the University campus.

The first group mentioned consisted of 10 trees obtained from Taylor's Nursery, which were known to them as European mountain ash. The exact origin of these trees was unknown because they were grown from seedlings obtained from seeds collected from the better trees found growing in Edmonton. The trees from Taylor's Nursery were of uniform growth, bark color and general appearance, and although their genetical background cannot be ascertained, they are of the same species, namely, *Sorbus aucuparia*. These trees were grown in 56-pound butter boxes in a 3:1 black loam sand mixture.

The mountain ash trees in Mr. Harcourt's orchard were also of the species *Sorbus aucuparia*, but had a more bushy growth habit than those from Taylor's Nursery. Bark color, leaf size and shape and other characters were the same in both groups.

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* The writer wishes to acknowledge his indebtedness to the Taylor Nurseries and to Mr. G. Harcourt for a generous supply of mountain ash trees which made possible much of this investigation.

The poplar trees inoculated were *Populus tremuloides* trees growing in their natural habitat near the western edge of the University of Alberta campus. Both young and old trees were inoculated.

The willow trees used were growing in the same location as the poplars, and were also in their natural habitat. The willow species was the same as that from which *Cytospora* isolations were made, namely, *Salix discolor*.

Bud Inoculations

Object.

In a study of the epidemiology of apple scab caused by *Venturia inequalis*. Keitt and Jones (8), in 1926, found "a striking relation between the stage of development of apple leaves and their susceptibility to scab". They found that tissues in freshly opening leaf buds and fruit buds were in the most susceptible stage, and that resistance increased with age.

Because it is probable that small wounds occur along the margin and base of the bud scales in the mountain ash when the buds are pushed open, it was thought that infection might occur when the buds were opening as Keitt and Jones found in the apple scab disease. It was reasoned that the occurrence of infection at this stage might be responsible for the commonly observed "Die-Back" condition which is characterized by the death of the limbs from the tip down.

Methods.

Buds of the mountain ash in various stages of development were inoculated with pycnospores or mycelial cultures during the spring of 1938. The work was done in the open during a spell of rainy weather and again during a warm spell. Spores were sprayed on the terminal buds with an atomizer, or mycelium was smeared on with a sterile scalpel, and some buds were left uncovered while some were wrapped in moist cotton and covered with glassine bags. The spores used were obtained from a mixture of cultures from the mountain ash numbers 2, 3, 10, 12 and 14 in one test, and a mixture of cultures from the poplar including numbers 1 to 7 in another. Mycelia of cultures 2 and 12 from mountain ash were applied by smearing buds following sterilization with 70% alcohol. The inoculated buds were then wrapped in moist cotton, covered with glassine bags and left undisturbed for a week. No fruit buds were available for this work.

Results.

Though numerous inoculations were made under varying weather conditions, there was no indication of leaf bud infection. This is perhaps significant in view of the fact that cultures 10 and 12, spores from which were included in leaf sprays, proved to be pathogenic in wound inoculations.

Flower Inoculations

Object.

Keitt and Jones, in their study of the epidemiology of apple scab, also found that apple trees could become diseased by the entrance of the causal organism, Venturia inequalis, through blossoms. That the mountain ash might become infected by the entrance of Cytospora spp. in a similar manner seemed probable. An experiment was outlined, therefore, to determine whether infection of the mountain ash occurred following flower inoculations with spores of Cytospora spp.

Methods.

Flower clusters on two mountain ash trees were inoculated by spraying spores on them with an atomizer on the evening of June 13, 1938. Because the weather was damp, cool and cloudy, it was thought that germination of the spores would take place readily and that, therefore, conditions for infection were very favorable.

Only 9 flower clusters were available, so a mixture of spores from isolates from mountain ash only was used. This mixture consisted of spores from cultures 2, 3, 10, 12 and 14, numbers 10 and 12 being of the Cytospora ambiens pathogenic group, and the others representing the fast growing, non-pathogenic group. Of the 9 clusters available, 6 were inoculated and 3 of these

were bagged with large paper bags to prevent drying out quickly on the morning following inoculation. The three left as checks were unbagged.

Results.

Results which are presented in Table I indicate that blossom infection may occur if the spores are deposited on the blossoms at the proper time. It was found, however, that an abscission layer was formed at the base of the pedicel and consequently the infected blossoms dropped off before the fungi could enter the tree. *Cytospora* was readily isolated from the infected carpels and pedicels.

TABLE I

Infection of blossoms of mountain ash following spraying with spores of *Cytospora*

Treatment	Cluster number	Total number of blossoms in cluster	Number of blossoms infected
Check	1	103	0
	4	87	0
	7	94	0
Inoculated unbagged	2	121	5
	5	83	5
	8	72	3
Inoculated bagged	3	90	2
	6	114	4
	9	78	3

The fact that blossoms may become infected may be an important contributing factor to the initiation of disease in a weak tree where the fungus could possibly gain entrance to the spur before an abscission layer could be formed.

Wound Inoculations

Object.

Former investigators, for example Schreiner (16), Povah (13), Long (11) and others, have reported *Cytospora* and *Valsa* species as being weak wound parasites and as gaining entrance to the host through injuries made by fire and other agents.

Wound inoculations were therefore made to determine the pathogenicity of the various isolates on poplars, willows and mountain ash.

Methods.

The cork borer method of Wright (18) was used for the first inoculations made in the spring of 1938. This method consists of removing a plug of bark from the tree (using sterile technique), placing some inoculum in the hole, replacing the plug and sealing with a band of adhesive tape.

It was thought that this method might be advantageous because the central plug would die and afford a favorable medium for the fungus to begin its development. However, out of 37 inoculations on the mountain ash with fungi isolated from diseased mountain ash, only three produced doubtfully positive infections. No infection occurred in the remainder of the 37 inoculations. The central plugs which had died had been invaded in all cases, and pycnidia were produced on many of these plugs from two to three weeks.

The failure of this method of inoculation is attributed to two factors. The first of these is the immediate stimulation of wound periderm around the wound, which occurs when the plug is removed. This layer may be able to inhibit the fungus from entering the host tissue. The second is the shrinking of the central plug as it dries, which leaves a space between it and the surrounding bark. Owing to this space, the fungus present is not in direct contact with the surrounding healthy bark.

The second method tried and adopted for all subsequent inoculations was that of making a ⊥ shaped slit in the bark, turning up the corners and placing the cultures in the wound. The corners died and afforded a point of entrance for the fungi which was still intact with the rest of the bark. It is not so likely in this case that a cork barrier would be formed behind the dead corners before the fungus began its progress through the dead bark. This method was found to be satisfactory, and the procedure was as follows:

The bark was surface sterilized with 70% ethyl alcohol where the inoculation was to be made and a ⊥ shaped wound was made with a sterile scalpel. The edges of the slit were turned up, inoculum (spores or mycelium) inserted and the wound was then covered with a piece of moistened sterile cotton batting. The cotton was then wrapped to the branch with a glassine bag which was wired below and above the wound, thus forming a sterile inoculation chamber.

More than 500 inoculations were made on poplars, willows and mountain ash. The results of the largest experiment are presented in Table II.

Results.

It will be observed from the results presented in Table II that isolates from the mountain ash numbered 7, 10, 11 and 12 were pathogenic on the mountain ash trees grown in boxes. These isolates formed the Cytospora ambiens group. Inoculations with Valsa ambiens in this experiment and in others also produced successful infection on the trees grown in boxes. No other cultures tested can be considered as pathogenic to the mountain ash.

Mountain ash trees growing in Mr. Harcourt's orchard failed to become infected with any of the cultures. This may possibly be attributed to the vigorous condition of these trees.

TABLE II

Degree of infection following wound inoculations with *Cytospora* species

Source of culture	Trees on which inoculations were made			
	Populus	*Salix*	*Sorbus* (Orchard)	*Sorbus* (Boxes)
	Average	Average	Average	Average
Check	0	0	0	0
Sorbus				
1	0	s	0	s
2	0	s	0	0
3	0	0	0	0
4	0	s	0	s
5	0	s	0	0
6	0	s	0	0
7	0	s	0	1
8	0	0	0	0
9	0	s	0	0
10	0	1	0	1
11	0	s	0	2
12	0	0	0	2
13	0	0	0	s
14	0	0	0	0
Populus				
1	0	s	0	0
2	1	1	0	0
3	1	1	0	0
4	2	0	0	0
5	s	1	0	0
6	2	1	0	0
7	s	1	0	0
Salix				
1	0	1	0	0
2	1	s	0	0
3	s	1	0	0
4	0	s	0	0
Prunus	0	s	0	0
V. ambiens	0	0	0	2

0 - no infection; s - slight infection, perhaps doubtful; 1 - small canker developing; 2 - canker developing and pycnidia present.

The willow seems to be more susceptible to attack by all species of *Cytospora* used than does either poplar or mountain ash. This is probably due to a natural susceptibility of this tree. The willows were growing under apparently normal conditions.

Poplars were susceptible to some of the cultures of *C. chrysosperma* isolated from poplar and to one isolate from willow. This isolate is believed to be *C. chrysosperma* also because of its close resemblance to the isolates from poplar in cultural character and spore size.

In general, the inoculation studies indicate that the *Cytospora* species used in these experiments are more pathogenic to the tree species from which they were isolated than they are to either of the other trees upon which they were tested. It was thought at the initiation of these studies that the *Cytospora* species associated with diseased poplars might be important as causal agents of the disease of mountain ash. These inoculation studies tend to disprove this theory. Other results of a search for possible relations that might exist between *C. chrysosperma* from the diseased poplars and the disease of the mountain ash are reported under "*Cytospora chrysosperma* in Relation to the Disease of the Mountain Ash".

AN EXPLANATION OF THE MANNER IN WHICH THE DISEASE PROGRESSES

The progress of the disease every season and the formation by the tree of a cork barrier, which retards this advance during the greater part of the year, has been described. The pathogenicity of Valsa ambiens and Cytospora ambiens has been proved by inoculation studies. By observing cankers carefully during the spring and summer of 1937 and 1938 it was found that they advance only during the spring and are walled off by a cork barrier during June, after which they remain dormant until the next spring.

A likely explanation of this progress of the canker during the spring only, is that the fungus concerned can grow in the spring before the tree has recovered from its winter dormancy when it apparently cannot produce a cork barrier to halt the advance of the fungus. At this time the fungus must pass the cork barrier which has held it in check during the previous season. It then kills a new zone of tissue. The fungus advances in the healthy bark until the tree, after recovering from its winter dormancy during the latter part of May or first part of June, produces a new cork barrier which halts the progress of the fungus for the season.

That the pathogen was alive in the cankers during the winter and therefore able to grow, was proved by isolations. These were made by removing and plating out infected tissue from areas adjacent to the cork barrier during the late winter and early spring. Isolations were also made from the healthy bark adjacent to the cankers during May, when it was found that on a number of diseased trees the bark in this region was turning brown and was showing signs of infection. Such tissues later died to form new parts of the cankers. The development of the disease in this manner is illustrated in Figure 4, and the branch shown in this picture was one of the ones used for the study of the advance of the cankers. During the early part of June, a new cork barrier was formed, and the progress of the disease was halted for the season.

STUDIES ON THE PHYSIOLOGY OF FUNGOUS ISOLATES

Species of Cytospora and Valsa were isolated from mountain ash, willows, poplars and saskatoon and studied in pure culture. Different physiological characters of the various isolates were compared in an attempt to determine any special characters or adaptations which they might possess individually or in common. This investigation included studies on cultural characters, temperature relations, spore germination and growth on different media. These various phases will be presented separately.

Cultural Characters

All isolates were grown on potato dextrose agar in petri plates, under uniform conditions, for a comparative study of their cultural characters. Potato dextrose agar was prepared according to Davis' formula (4). Cultures were incubated uniformly at room temperature (20°C. to 25°C.).

Isolations from the Mountain Ash.

Isolates of Cytospora from the mountain ash fall into two groups as to cultural characters. These groups have already been described (page 15).

Isolations from Poplar.

The isolates from poplar formed a single group of uniform appearance and growth. The colonies were characterized by a brownish mycelium, rough to lobed margins, and more aerial mycelium than the isolates from mountain ash. The fungus represented by these isolates has been identified by Dr. J. Dearness as *Cytospora chrysosperma*. The growth rate of these isolates is approximately the same as that of members of the fast growing group from mountain ash. Representative isolates from mountain ash and poplar may be compared in Figure 15.

Valsa sordida, reported as the perfect stage of *C. chrysosperma* by Schreiner (16), was also found on several of the diseased poplars examined, and was plated out for comparison with *C. chrysosperma*. Colonies of *V. sordida* on potato dextrose agar showed a type of growth very similar to *C. chrysosperma*, but the mycelium was greyish to grey green, turning dark green to black with age. Both *V. sordida* and *C. chrysosperma* showed some variability in type of growth and ability to produce pycnidia.

Isolations from Willow.

The isolates from willow constitute a rough light grey group characterized by colonies with lobed edges, brownish or greenish mycelium and irregular growth. In general, these cultures resemble closely the cultures of *C. chrysosperma* from poplar.

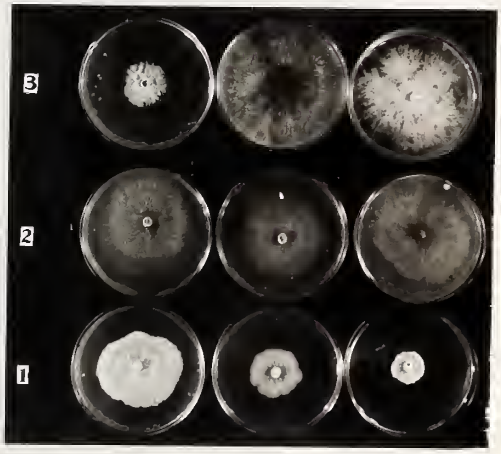


Figure 15

Cultural characters of Cytosporae from poplar and mountain ash

1. Valsa ambiens from mountain ash, slow growing with white mycelium.

2. Cytospora cincta (?) from mountain ash, fast growing with green mycelium.

3. C. chrysosperma from poplar, grey to brown mycelium with rough colonies.

Differences from left to right represent differences between isolates within a species.

Isolations from the Saskatoon.

All specimens of diseased saskatoon twigs examined harboured a Valsa which has been identified tentatively by Dr. Dearness as "at or near Valsa cincta Fr.". Isolates of this fungus grown in petri dishes on potato dextrose agar could not be differentiated by cultural characters from those of the fast growing group from the mountain ash. The mycelium was greenish and the colonies grew rapidly and had smooth edges. Little aerial mycelium was produced.

The characteristic color of isolates from all hosts was produced in from 5 to 7 days at 25°C. Pycnidia were produced in all isolates after from 3-4 weeks at room temperature (approximately 25°C.), and they appeared as hard aggregates of black hyphae half embedded in the agar and protruding as small black elevations in the mycelium.

Pycnospores were produced in large quantities and usually appeared as globules on the summits of the pycnidia. However, if the atmosphere in the plates was dry, typical spore horns were formed. Pycnospores produced by cultures grown on potato dextrose agar were of the same size and regularity as spores produced in nature on the host. Spore dimensions of the various isolates are given in Table III.

TABLE III

Pycnospore size of different isolates in microns

	Length		Width average
	Limits	Average	
1. _C_. _ambiens_ slow growing group on mountain ash	5.0 - 6.9	5.8	1 - 1.3
2. _C_. _cincta_ (?) fast growing group on mountain ash	5.0 - 6.2	5.6	1 - 1.2
3. _C_. _cincta_ (?) from Amelanchier	5.0 - 6.2	5.7	1 - 1.3
4. _C_. _chrysosperma_	3.1 - 4.6	3.8	1.0

Characteristics of individual isolates have remained constant for two years in culture with no indication of saltations or variations of any kind.

Effect of Temperature on Growth

Object.

Studies of the effect of temperature on growth were undertaken in order (1) to obtain a better understanding of the development of the disease as it occurs in the spring when low temperatures usually prevail; (2) to produce another basis of differentiation of the various groups of fungi under investigation.

Methods.

The effect of temperature on the growth rate of groups of isolates from the different hosts was determined by measuring the growth rate of colonies cultured on potato dextrose agar at different temperatures. Petri plates were sown in the centre with uniform discs cut, with a 3/16" cork-borer, using sterile technique, from the edge of growing colonies of the various isolates. These discs were always placed with the mycelium next to the agar of the plates to which they were added, so that all cultures would have an equal start. These plates were then incubated in thermostatically controlled incubators at temperatures of 3°, 6°, 15°, 20°, 24°, 30° and 35°. Measurements of the diameter of the colonies were made each day for seven days, at which time most of the rapid growing isolates had completely covered the plates.

In these studies the 15 isolates from mountain ash, 7 isolates from poplar and 4 isolates from willow that were used in inoculation studies, were tested. As has been described, isolates numbered 7, 10, 11 and 12 from the mountain ash were slow growing strains of the C. ambiens group, and the other 11 of the 15 were fast growing cultures of the other group. There were, therefore, 4 groups, two from the mountain ash and one each from willow and poplar. Members of each group were considered as replicates for purposes of statistical analysis. The graph, Figure 16, is constructed from average measurements of growth in diameter of colonies for each group after 6 days.

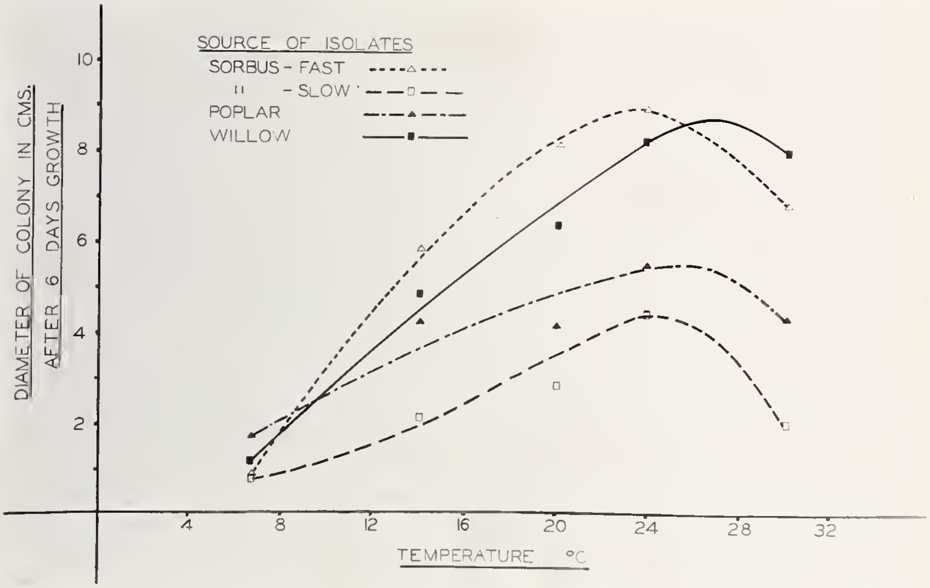


Figure 16

The effect of temperature on the growth of *Cytospora* spp.

Results.

The data presented indicate that the optimum temperature for growth of all of the strains studied is near 24°C. At 30°C. growth becomes irregular, and at 35°C. very little growth occurs. The fungi grow slowly at 6°C., but at 3°C. no growth occurred even after three weeks. However, when the plates were moved from the 3°C. to the 20°C. incubator normal growth commenced. This indicates that the fungi were not harmed by the low temperature. The growth of all cultures at 14°C. is comparatively rapid.

These temperature studies indicate that the fungi can grow quite well at temperatures which prevail at Edmonton during the early spring. The fungi can become active about the first of April when temperatures frequently reach as high as 18°C. during the day and light frosts occur at night. The tree does not become fully active until the latter part of May, which apparently would allow the fungi approximately a month to grow before they are walled off by cork barriers laid down by the tree. Temperature studies therefore support the hypothesis that the progress of the disease on the individual trees of mountain ash during this period might be due to the growth of the fungi before the tree has recovered sufficiently from its winter dormancy to actively lay down a retarding cork barrier.

That fungi of all of the Cytospora species tested might be of importance in relation to the progress of

the die-back disease on their respective hosts during the spring is indicated by these temperature studies.

Spore Germination Studies

Object.

Spore germination studies were made for the purpose of (1) determining the manner in which spores of the Cytospora species concerned germinate; (2) comparing the germination of the spores of the different species on various media; and (3) determining the effect of different temperatures on spore germination. Spores of isolates from willow, poplar and mountain ash were included in these studies.

Methods.

In order to compare the germination of spores from the various isolates, hanging drops of 2% dextrose solution in Van Tieghem cells were used. These drops were then incubated uniformly at 20°C. and examined frequently under the microscope. For comparison of the effect of different media and different temperatures on spore germination, hanging drops of extracts from poplar, willow and mountain ash were sown with spores and incubated at temperatures ranging from 6°C. to 35°C.

Germination on 2% dextrose solution.

The pycnospores of isolates from poplars, willows and mountain ash germinated well on 2% dextrose solution, and the first observations of spore germination were made on this medium. The germination of pycnospores from both groups isolated from the mountain ash was essentially the same. No difference in spore size or method of germination could be detected. A photograph of germinating spores of isolate No. 2 from mountain ash (fast growing group) is presented in Figure 17. The most striking feature is the enormous size to which the spores swell before a germ tube is sent out. The small spores that can be seen in their natural dormant condition in Figure 17 are about 1 x 6 microns, and they swell up when germinating to 6 x 9 microns before a germ tube is produced. This represents a volume increase of from 46 to 50 times, and an expansion of the spore wall from approximately 19 square microns to 180 square microns. It would be interesting to know whether the spore wall stretches or is built up during this expansion.

At 20°C. the first germ tubes from pycnospores of mountain ash isolates were observed after 20 to 24 hours on 2% dextrose. A germ tube is first put out from one end and another may be produced on the other end or it may not. Germination was most frequently from one end only.

Spores from poplar isolates swelled to only half the size of spores from mountain ash isolates. This difference can be seen by comparing Figures 18 and 19

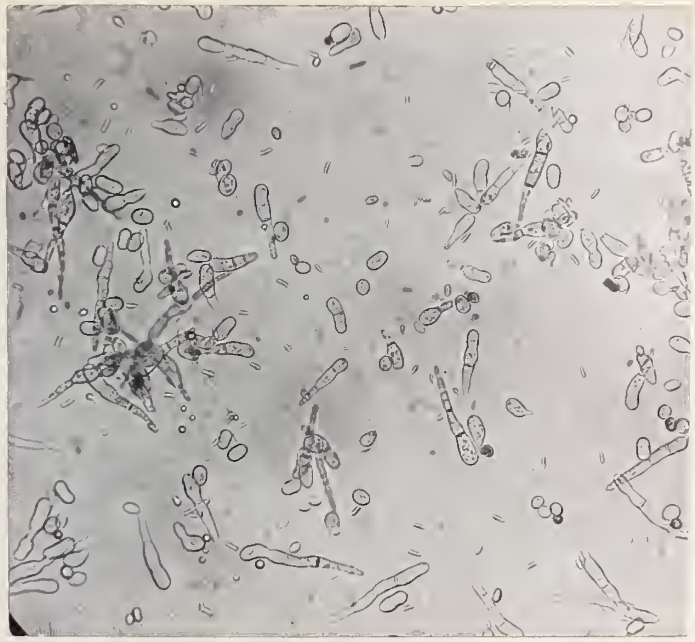
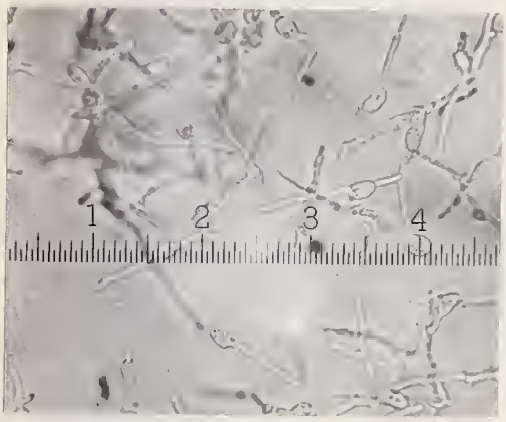


Figure 17

Germinating spores of fast growing type from mountain ash showing enormous swelling and germination at various stages. (Magnification 280 times)



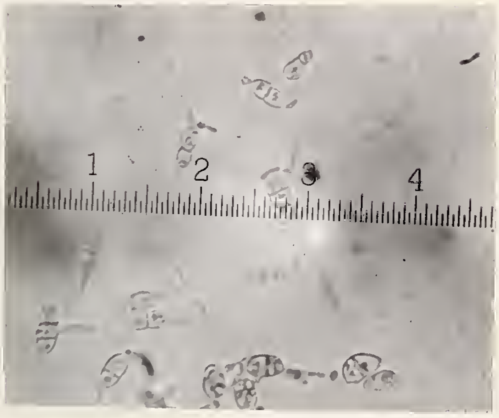


Figure 18

Germinating spores of C. chrysosperma (Magnification 497 times)

Figure 19

Germinating pycnospores of C. ambiens on dried drop of 2% dextrose showing production of secondary conidia and septations (Magnification 497 times)

which are of the same magnification. It is to be remembered, however, that spores from poplar isolates are naturally smaller (see Table III). Another notable contrast between germination of spores from poplar isolates and those from mountain ash isolates is the frequent occurrence of 1-3 septations in the spores of mountain ash isolates of both groups soon after they start to swell (Figures 19 and 20). These septations have not been observed in germinating spores from willow and poplar isolates.

Spore germination on different media.

A comparison of the rapidity of spore germination on different media was undertaken with the object of differentiating the various isolates with respect to their readiness to germinate on extracts from different trees.

Media were made by extracting 10 grams of bark of poplar, willow and mountain ash, respectively, with 100 cc. of a NaOH, KH_2PO_4 buffer at pH 6.2. Hanging drops of sterile buffer and of extracts were sown with spores from mountain ash, willow and poplar isolates and placed in thermostatically controlled chambers at temperatures from $6^{o}C$. to $35^{o}C$. The drops were examined under the microscope after 24 and 48 hours. Results of a typical spore germination experiment are presented in Table IV.

Spores germinated in from 18 to 24 hours at $24^{o}C$. which proved to be the optimum temperature for germination. Germination required 48 to 60 hours at $14^{o}C$. and was very slow and erratic at $6^{o}C$. Germination of some spores in each

TABLE IV

The germination of spores from mountain ash and poplar isolates at different temperatures on different media

		Spores from mountain ash isolates								Spores from poplar isolates							
Medium		Mountain Ash		Willow		Poplar		Buffer		Mountain Ash		Willow		Poplar		Buffer	
Hours germinated		24	48	24	48	24	48	24	48	24	48	24	48	24	48	24	48
Temperature	6°C.	no sw	sw*	no sw	sw	no sw	no sw	no sw	sw	no sw	sw	no sw	sw	no sw	no sw	no sw	sw
	14°C.	sw & g.t.	g.t.+	sw & g.t.	g.t.	no sw	no sw	sw	g.t.	sw	sw & g.t.	sw	sw	sw	sw	sw	sw
	20°C.	sw & g.t.	g.t.	sw & g.t.	g.t.	no sw	sw	sw & g.t.	g.t.	sw & g.t.	g.t.	sw & g.t.	g.t.	sw	sw & g.t.	sw & g.t.	sw & g.t.
	24°C.	g.t.	g.t.	g.t.	g.t.	sw	sw	sw & g.t.	g.t.	sw & g.t.	g.t.	sw & g.t.	g.t.	sw & g.t.	g.t.	sw & g.t.	g.t.
	30°C.	sw & g.t.	sw & g.t.	sw & g.t.	sw & g.t.	sw	sw	sw & g.t.	sw & g.t.	sw	sw & g.t.	sw	sw & g.t.	sw	sw & g.t.	sw	sw & g.t.
	35°C.	sw	sw & g.t.	sw	sw & g.t.	sw	sw	sw	sw	sw	sw & g.t.	sw	sw	sw	sw & g.t.	sw	sw

* sw - swelled.

+ g.t. - germ tubes produced.

drop had taken place in 24 hours at 30°C. in most cases, but many remained dormant. At 35°C. the majority of the spores either remained dormant or swelled without germinating.

Comparison of germination on the different tree decoctions shows that spores germinate vigorously on mountain ash and willow decoctions, and not so well on buffer solution alone. However, only spores from poplar isolates germinated up to the end of 48 hours on the poplar decoction medium. Poplar extract apparently contains some ingredient which tends to prevent the germination of spores from willow and mountain ash isolates. Spores of the poplar isolates C. chrysosperma are not able to germinate on the poplar decoction as well as on the willow and mountain ash decoctions.

During these studies an interesting phenomenon was noticed in that when spores of mountain ash isolates of both groups had germinated and then the drops had been allowed to dry, small secondary conidia were formed in whorls along the sides of the hyphae (Figure 21). In some cases these secondary conidia were formed directly as a whorl on the end of the spores with only a short stalk or no stalk having been produced (Figure 19).

These secondary conidia measure 5-6 microns long, so are almost as large as the regular pycnospores. Their germination has not been observed, but they probably function in the same manner as pycnospores, and they are apparently only produced when conditions are unfavorable

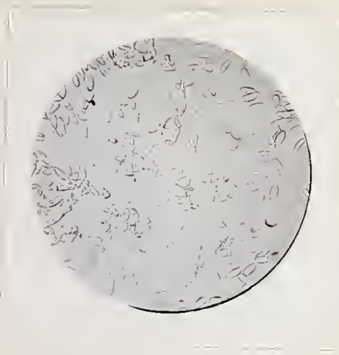


Figure 20

Germinating spores of _C_. _ambiens_ showing production of septations during swelling (Magnification 142 times)

Figure 21

Hypha of _C_. _ambiens_ on dried drop showing whorls of secondary conidia (Magnification 142 times)

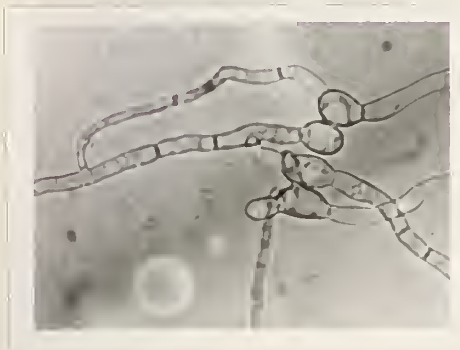


Figure 22

Germinating pycnospores of _C_. _ambiens_ showing anastomoses (Magnification 497 times)

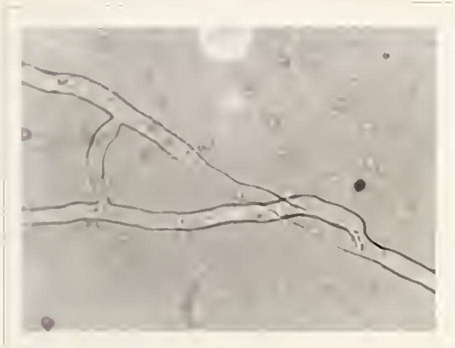


Figure 23

Hypha of _C_. _ambiens_ showing intra-mycelial fusion (Magnification 497 times)

such as when the hanging drop dries up. They have never been observed in connection with germinating spores on agar.

Another important difference between spores of isolates from mountain ash and those of isolates from poplar has been observed. Fusions or anastomoses frequently occur between hyphae produced by the germinating spores of mountain ash isolates, but they have not been observed in C. chrysosperma from poplars. These fusions occur between hyphae from different spores (Figure 22) and between branches of a hypha from the same spore (Figure 23). The significance of these fusions has not been determined.

Growth Rate on Different Media

Object.

Inoculation studies indicate that species of Cytospora are, in general, only pathogenic on the species of tree from which they were isolated. Spore germination studies showed that only spores of C. chrysosperma can germinate on poplar decoction medium.

Because the above-mentioned studies indicate that Cytosporeae from each of the three different trees do not readily infect the other two trees, and that spores of Cytospora spp. isolated from willows and mountain ash are inhibited in some physiologic manner from germinating on poplar extract, experiments were undertaken to test the ability of the different isolates to grow on extracts from each of the three tree species.

Methods.

A small experiment was outlined in which representative isolates from poplar, willow and mountain ash were grown on separate media containing extracts from each of these trees. The extracts were made from finely ground twigs and bark. One hundred grams of this material were extracted with 1000 cc. of cold water for 12 hours. The extract was then filtered through Number 10 Whatman filter paper, and made up to 1 litre. The final medium was made by adding 20 grams of agar agar, and 50 grams of

dextrose to this extract and autoclaving the mixture for 15 minutes at 15 pounds pressure.

The same technique as was used in the temperature studies was employed in determining the relative growth rates of the fungi on the different media. The figures given in Table V represent the average diameters of the colonies after 7 days growth. Each figure is an average of 10 replicates.

TABLE V

Growth of Cytosporeae on mountain ash, poplar and willow decoction agar after 7 days

Media	Average diameter of colonies in cm.		
	Source of cultures		
	Mountain Ash	Willow	Poplar
Mountain Ash	5.25	5.9	5.2
Willow	3.20	5.8	6.3
Poplar	0.0	0.0	3.0
Minimum significant difference	0.23	0.21	0.27

Results.

These results presented in Table V indicate that C. chrysosperma, the isolate from poplar, grows readily on extracts from all three trees, although its growth is retarded somewhat on the extract from poplar. Isolates from mountain ash and willow, on the other hand, grow only on extracts from the mountain ash and willow and not on the extract from poplar.

It was thought that the failure of the mountain ash and willow isolates to grow on the poplar decoction medium might be due to an unfavorable pH. Potentiometer determinations of the pH of the media were therefore made. These showed that the poplar decoction had a pH of about 5.4, while the pH of the mountain ash and willow decoctions was approximately 6.2 in each case.

To further elucidate this phase of the problem, an attempt was made to find the optimum pH for each group of isolates, and at the same time find a pH at which all media could be buffered during later experiments so that one could be certain that the reaction of the medium would not inhibit growth of any of the isolates.

A series of McIlvaine's standard buffers of pH, 4.0, 5.0, 6.0, 7.0 and 8.0 were made up, diluted 1-10 and used as the liquid in potato dextrose agars. It was found, however, that the cultures would grow only in the medium at pH 4.0 on this buffer, and it was suspected that the ingredients of the buffer itself (citric acid and Na_2HPO_4), rather than the pH, prevented growth at higher pH levels. The concentration of Na_2HPO_4 at the higher pH values seemed to be toxic.

Another experiment was then outlined using the following Clark and Lubs standard buffers: (a) NaOH and KH phthalate for pH 4.2, 5.0 and 6.0 and NaOH and KH_2PO_4 for pH 6.0, 7.0 and 7.8. These buffers were diluted 1-10 and used as the liquid in potato dextrose agar as before. In this experiment four isolates of the slow growing group

from mountain ash, five isolates from the fast growing group, four isolates from poplar and three isolates from willow were used, and each member of each group was treated as a replicate for purposes of statistical analysis. The results are presented in Table V and illustrated by the graph in Figure 24. These results indicate that all isolates will grow over quite a pH range from 4.2 to 7.0, and that the optimum for most cultures probably lies near 6.0. Here again though, it is noticeable that a reduction in growth occurs with a change in concentration of the ingredients of the buffer, independently of pH. Otherwise the curves would coincide at pH 6.0. It will also be noted that, as the Na_2HPO_4 is increased, using McIlvaine's buffer, the growth of the fungi is inhibited, while if the other two buffers are used the growth of the fungi is retarded as NaOH is increased. Because sodium is a common substance in both of the compounds that are increased, it appears that sodium is toxic to the growth of Cytospora.

TABLE VI

Growth rate of Cytospora isolates on different media.

Medium	Average diameters of colonies after 3 days in cm.				
	Source of cultures				
	Sorbus		Willow	Poplar	Sorbus Culture No. 17
	Fast	Slow			
Sorbus	6.3	2.6	5.3	3.8	6.2
Willow	2.6	2.0	4.7	4.2	4.9
Poplar	0.8	0.9	2.1	3.0	4.0
	0.0	0.0	0.0	0.0	0.0
Min. sig. difference	.18	.24	.19	.25	

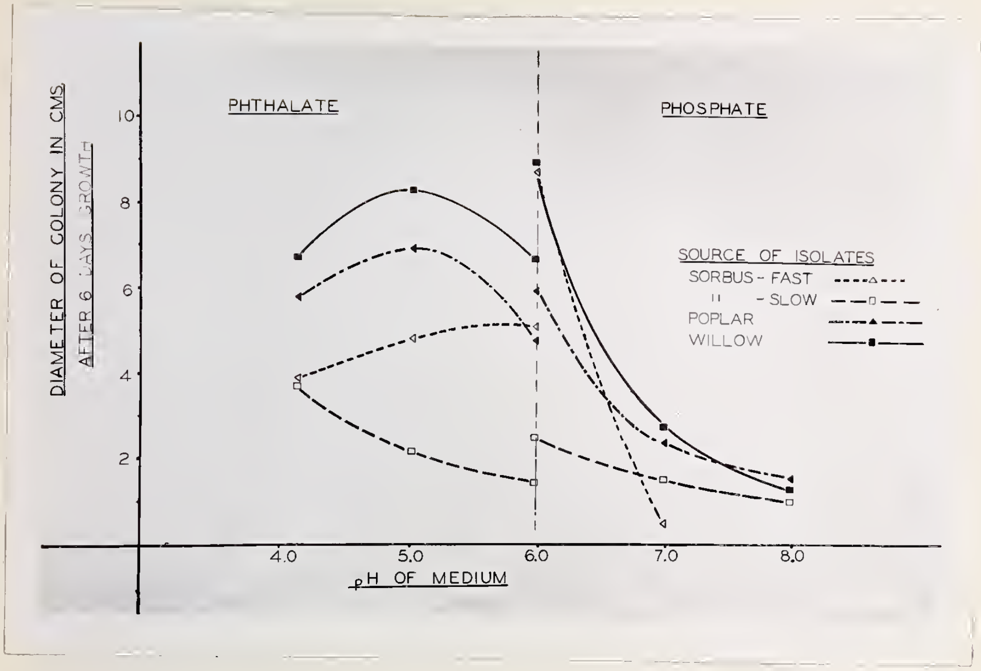


Figure 24

The effect of the reaction of the medium on the growth rate of Cytosporeae

NaOH and KH phthalate was used as buffer from pH 4.2 to 6.0

NaOH and KH_2PO_4 was used as buffer from pH 6.0 to 8.0

Though these studies were not very successful in determining the exact optimum pH for the growth of the fungi, they indicated that the media could be buffered at pH 6.0 with a phosphate NaOH buffer. At this pH satisfactory growth of all of the fungi under consideration could be obtained.

A larger experiment on the growth on different media was then conducted. The media used in this experiment were buffered at pH 6.2 using a KH_2PO_4, NaOH buffer. In this experiment over 20 isolates were tested as to their ability to grow on buffered extracts from mountain ash, poplars and willows.

Five isolates from each host were used, and for purposes of statistical analysis and of obtaining the average growth rates presented in Table VI each isolate was considered as a replicate of the sample from the host from which it was obtained.

From the results presented in Table VI it will be observed that though the media were buffered as explained, isolates from mountain ash were able to make but little growth on the poplar decoction medium. The isolates of _C_. _chrysosperma_ from poplar, on the other hand, grew quite well on both the poplar and mountain ash decoction media.

In view of the fact that similar differences were noted in spore germination studies, it seems that

C. chrysosperma must have some physiological quality which enables it to grow on the poplar decoction agar and on poplar trees. This evidently is lacking in the other species of Cytospora examined in these studies.

The fact that C. chrysosperma has the ability to grow on poplar, however, does not mean that it cannot grow on the mountain ash. In fact, when growth rates and spore germination on the different media are compared, it is observed that C. chrysosperma grows better on the mountain ash decoctions than on those from poplar. This would indicate that unless other factors interfered, C. chrysosperma could change hosts, from the poplar to the mountain ash, quite readily. Evidence will be presented later, however, that indicates the existence of limiting factors which tend to inhibit the growth of C. chrysosperma on mountain ash.

THE SOURCES OF FUNGI ASSOCIATED WITH THE "DIE-BACK" OF MOUNTAIN ASH

The Fast Growing Group

C. chrysosperma apparently occurs very rarely on the mountain ash. In the course of these studies it was found only once, and then only on a small dead twig. Since this was the only Cytospora isolated from poplars, it would appear that there is no important relation between the die-back diseases of these two trees. These two diseases are evidently distinct and caused by different species of Cytospora.

Isolates of Cytospora from willows also differed from those obtained from the mountain ash. It would appear, therefore, that both poplars and willows may be disregarded as sources of inoculum for the die-back disease of the mountain ash.

Upon the elimination of poplars and willows as sources of inoculum, a fresh search was begun for less common trees and shrubs harbouring Cytosporeae. As has been mentioned elsewhere in this paper, it was found that cultures on potato dextrose agar of a Valsa found on the saskatoon (Amelanchier alnifolia), a very common shrub in Edmonton, were almost identical with cultures of the fast growing Cytospora isolated from mountain ash. Pycnospores of these two groups were measured and found to differ but

little in size. Those from the fast growing mountain ash group averaged 5.6 microns in length, while those from the saskatoon averaged 5.7 microns. That the same fungus could be common on these two trees is perhaps to be expected in view of their close relationship. It is of interest to note in this connection that a natural cross between them, named *Amelosorbus jackii*, has been reported by Professor Rehder in Idaho.

When mountain ash twigs, in sterile flasks, were inoculated with *Valsa* from the saskatoon, pycnidia, spores and spore horns almost identical with those of the fast growing isolates from the mountain ash were obtained. Dr. J. Dearness identified the *Valsa* as "at or near *Valsa cincta*", but as yet he has been unable to say whether the *Valsa* from the saskatoon is identical with isolates of the fast growing group from the mountain ash. It seems, however, from the similarity of all physiological and morphological characters examined, that these isolates from the saskatoon and mountain ash are of the same fungus. Evidence accumulated thus far indicates that if the isolates in question are of the same species, then this species is capable of transferring from the saskatoon to the mountain ash. If it does not actually transfer, at least it is common to the two and is present as a *Valsa* on the saskatoon and as a *Cytospora* on the mountain ash. However, neither the *Valsa* from the saskatoon nor the fast growing *Cytospora* from the mountain ash has been found pathogenic to the mountain ash.

The Valsa ambiens Group

Neither Valsa ambiens nor its imperfect stage Cytospora ambiens was found on any of the native trees or shrubs examined. It was thought possible, however, that this species might be present on the native mountain ash (Sorbus americana) which grows in the foot-hills and in the more northern parts of the province. It has been reported that some trees of the native species have been brought into this city and transplanted on lawns as ornamentals. It seemed reasonable that if trees that were brought in harboured Valsa or Cytospora ambiens, then they might constitute a source of inoculum from which the European mountain ash might have become infected.

Following up this possibility, a letter was sent to Mr. W. D. Albright* of the Dominion Government Experimental Station at Beaverlodge, Alberta, asking him for information on, and for specimens of, the native mountain ash which is quite common around Beaverlodge. In reply to this letter Mr. Albright reported that the native and European species of mountain ash were under observation at his station where they are planted alternately in a row along a windbreak for comparison with each other. Mr. Albright's observations on the two species indicate that the native mountain ash grows slowly with a

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* The writer wishes to acknowledge information and valuable suggestions received from Mr. W. D. Albright, Superintendent, Dominion Experimental Station, Beaverlodge.

bush-like habit and is very hardy, while the European mountain ash grows rapidly, is more tree-like and is susceptible to die-back, to winter killing and to sunscald. No disease could be found on the native trees, but samples were sent of diseased branches from the European species. Cytospora of both the Cytospora ambiens and the fast growing group common in Edmonton were isolated from these twigs.

Though no other native mountain ash trees have been examined, observations made on the trees at Beaverlodge indicate that the native mountain ash does not generally harbour Valsa or Cytospora species. The native species may be resistant to, and free from infection by, Valsa and Cytospora species, and if so, it is not likely to be a source of the inoculum. The source of members of the Valsa and Cytospora ambiens group has therefore not been determined. This fungus may possibly have been introduced with nursery stock of the European species.

PREDISPOSING FACTORS IN RELATION TO "DIE-BACK" OF MOUNTAIN ASH

Though Valsa and Cytospora species are always associated with the die-back of mountain ash, and though some of these species have been proved to be pathogenic, factors other than the presence of these fungi are thought to be more important in the initiation of the disease. It is believed that the tree must become predisposed before infection by Valsa or Cytospora will occur. Facts observed that support this belief may be briefly outlined as follows:

(1) On 78 percent of the diseased trees examined only the non-pathogenic fast growing species of Cytospora was present. The fact that this fungus grows quite well on dead twigs but is not pathogenic suggests that the limbs on which it is found have either died or were weakened or injured by agents other than the fungus before infection occurred. Assuming that the tree must be in a weakened condition before infection occurs, then the reason for the domination of the non-pathogenic species is quite clear. As has been shown, the non-pathogenic species seems to be present on the native saskatoon as well as on the majority of the diseased mountain ash, so when a tree becomes weakened or a branch dies there is a high probability that spores of this species will be present in a favorable position to cause infection. The pathogenic species, on the other hand, being found only on relatively few

mountain ash trees and on no other common host, is in the minority, and is not so liable to be present to infect a weakened tree. The pathogenic species is, however, apt to cause more serious infection of relatively healthy trees if it gains entrance through wounds or sunscald cankers.

(2) Many of the trees that were severely diseased were known to be exposed to adverse environmental conditions. Three severely infected trees were growing on the south edges of terraced lawns where the soil was dried out by the hot sun and by drainage. Lack of sufficient water had, in all probability, weakened these trees to such an extent that they could offer little resistance to invasion by fungi. Several diseased trees were growing close to caragana hedges (Figure 2), and there is no doubt that these trees were suffering from lack of moisture and nutrients due to competition with the hedges and were, therefore, in a weakened condition.

It is noteworthy also that in the inoculation studies only trees growing in boxes became infected. The trees growing in the orchard under more natural conditions were resistant to the fungi. The trees in the boxes were probably less resistant because of their confined root space, possibly also because of root injury sustained in transplanting.

Such observations suggest that the trees must be in a weakened condition before they can be attacked by Cytospora.

(3) Genetic factors are probably also of importance in determining resistance to die-back. Several cases have been observed in the course of these studies where only one tree out of two or three growing in the same yard, apparently under identical conditions, was diseased. It is believed that the diseased trees in these cases lack one or several genetic factors possessed by their companions which determine resistance. Further evidence in support of this theory is the fact that Dr. J. S. Shoemaker of the Department of Horticulture, University of Alberta, and the author of this paper have observed, independently of one another, that certain forms of mountain ash trees of the species Sorbus aucuparia are more susceptible to sunscald than others. Those with dark bark seem to suffer more from sunscald than those with light bark. Die-back also seems to be more severe in trees with dark bark. Dark and light bark are no doubt expressions of different genetic complexities, and this is evidence that resistance to sunscald and die-back may also be looked upon as being governed by genetic factors which are also apparently related to bark color.

It is concluded from these studies that trees must either be inherently susceptible or that they must become predisposed due to injury or to adverse environmental conditions before disease will occur. Fungi belonging to the genera Valsa and Cytospora are important, however, in that they attack and cause the die-back disease on weakened trees which otherwise might have survived the adverse conditions. These conclusions are supported by the work of Long (11), Povah (13) and Sanford (15).

SUGGESTED PREVENTIVE MEASURES

Because the disease seems to be influenced mainly by factors causing the predisposition of the trees, it appears that preventive measures based largely on relations of the disease to the environment and on proper selection and care of trees would offer most promise. In view of the fact that the disease is not epiphytotic in nature and is not associated with a vigorous pathogen, such control measures as spraying and dusting, which are used in the prevention of many tree diseases, are likely to be of little value.

The following preventive measures listed in order of their importance are suggested:

(1) The selection of trees for parent stock that are apparently resistant and which at the same time have qualities which make them desirable for ornamental purposes. Some of the European mountain ash trees observed during the course of these studies seem to be resistant to both sunscald and die-back, and at the same time are excellent ornamental trees. These trees could well be used as parent material.

Propagation by cuttings would be an efficient method of multiplication. It would be desirable to use cuttings because the progeny of a single tree would then be the same as the parent, genetically, and should therefore all be as resistant as the parent and possess its other desirable qualities. The recent introduction

of hormones for rooting cuttings makes the use of cuttings feasible. Commercial hormone solutions have proved very satisfactory for rooting cuttings used in this study*.

(2) The location of trees in relation to hedges, terraced ground, etc., appears to be important. It has been observed that many mountain ash trees growing near the edge of terraced ground or on southern slopes are severely diseased, while evergreens in like positions are apparently quite healthy. Evergreens grown in Edmonton seem to be able to survive quite well under conditions that are apparently too dry for the mountain ash. It can be recommended, therefore, that in landscape gardening the mountain ash should be planted singly and in the more favorable positions, while more hardy trees should be placed in the less favorable positions.

(3) In caring for the trees an adequate and regular supply of water should be provided. Very little watering may be required during average seasons, but in dry years application of enough water to keep the trees in a normal growing condition is advisable. It is thought that most cases of disease are initiated during dry years when branch tips or branches become weakened or die due to drought, and are then subject to attack by the Valsa or Cytospora species which have been described.

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* The writer is indebted to Mr. C. R. Ure of the Department of Horticulture, University of Alberta, for providing mountain ash cuttings rooted by means of hormone solution.

(4) Tree surgery is likely to prove a very valuable practice in helping to control this disease. Early excission of diseased branches is desirable to prevent spread of the causal fungi down the branches and to remove the infected tissue bearing inoculum for other weakened branches or trees.

Branches that are to be removed should be severed just above a side branch or bud, or close to the main trunk. If this is done no dead end will be left to become reinfected. It is desirable to paint the severed ends with asphalt paint, or similar substance, to prevent the entrance of disease producing organisms.

In view of the results of these studies as a whole, it is felt that if trees are selected carefully, and if the preventive methods as outlined above are observed, the European mountain ash can be grown quite successfully in Edmonton.

SUMMARY

1. Studies on a die-back disease of the European mountain ash are reported in this paper, and the signs and symptoms are described.

2. Most of the European mountain ash trees grown in Edmonton are affected. Some are severely damaged, but in the majority of cases only minor infection occurs.

3. Fungi belonging to the genera *Valsa* and *Cytospora* are always found associated with this disease.

4. *Valsa ambiens*, the only species of *Valsa* commonly associated with this die-back, is accompanied by its conidial stage, *Cytospora ambiens*. This fungus, however, is not especially common on the mountain ash.

5. A second fungus, isolated more frequently than *Valsa* or *Cytospora ambiens* from diseased mountain ash, is believed to be *Cytospora cincta*. Evidence has been obtained which indicates that *Valsa cincta*, the perfect stage of *Cytospora cincta* which is found on the mountain ash, is present on dead branches of the native saskatoon (*Amelanchier alnifolia*).

6. Only *Valsa ambiens* and *Cytospora ambiens* proved to be pathogenic to the mountain ash.

7. Cytospora chrysosperma, the only Cytospora isolated from diseased poplars, was found on the mountain ash only once. This would indicate that poplars affected with the die-back disease do not serve as sources of inoculum for the die-back of mountain ash.

8. Physiological studies indicate that the pycnospores of Cytospora ambiens, C. chrysosperma and C. cincta (?) germinate, and their mycelia grow readily, at temperatures ranging from 6° to 30°C., having an optimum of approximately 24°C.

9. Cytospora chrysosperma has been found to be the only fungus of those studied which will grow on extract of poplar (P. tremuloides) twigs. This is apparently due to the tolerance of this fungus to some ingredient in the poplar extract which inhibits the growth of the other species of Cytospora studied. The pH of the extract did not account for this inhibition.

10. The following methods for the prevention of this disease, based on observational and experimental evidence reported in this paper, are suggested:

(a) Selection of resistant trees and propagation of these trees by vegetative means.

(b) The planting of mountain ash only in locations favorable for their growth.

(c) Adequate and regular watering.

(d) Tree surgery. The removal of diseased branches is desirable because the disease may work farther into the tree, and because these branches are a source of inoculum from which other infections may occur.

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